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TITLE OF THE INVENTION (280 characters max)

METHODS FOR EX-VIVO AND IN-VIVO EXPANDING STEM/PROGENITOR CELLS

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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Respectfully submitted,

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METHODS FOR EX-VIVO AND IN-VIVO EXPANDING STEM/PROGENITOR CELLS

Inventors: Arik HASSON and Frida GRYNSPAN

Introduction

The ex vivo expansion of hematopoietic stem cells (HSC), Mesenchymal stem cells (MSC) and endothelial progenitor cells (EPC) are among the most challenging fields in cellular biotechnology. This is a rapidly growing area of tissue engineering with many potential applications in bone marrow transplantation, transfusion medicine, regenerative medicine or gene therapy. Over the last few years much progress has been made in understanding HSC, MSC, or EPC differentiation, discovery of cytokines, isolation and identification of cellular subtypes and in the development of a variety of bioreactor and supporting scaffolds concepts. All this has led to a number of (preliminary) clinical trials that gave a hint of the benefits that can be obtained from the use of expanded hematopoietic cells (Hoffman et al. 1993; Wagner 1993; Andrews et al. 1994; Purdy et al. 1995; Gehling et al. 1997; Bachier et al. 1999; Chabannon et al. 1999a; McNiece et al. 1999; Nielsen 1999; McNiece et al. 2000a; McNiece and Briddell 2001; Noll et al. 2002; Wolff 2002) MSC (Knutsen et al. 1998; Vilquin et al. 2002) or EPC (Chachques et al. 2002; Menasche 2002; Murohara 2003) in cellular therapy . Moreover, as the understanding of the complexity and the functionality of HSC, MSC or EPC transplantations in either clinical trials or in vivo animal models grows, it becomes obvious that the number and quality of cells transplanted per body volume/weight is crucial. Higher number of cells results with better therapeutic outcome (Bensinger et al. 1996b; Chown et al. 1996; Shizuru et al. 1996; Chabannon et al. 1999b; Barker and Wagner 2002; Jaroscak et al. 2003a). Cord bloods were found as a reach source for HSC transplantation but the low number of HSC cells collected in each cord blood unit limit their common use to children and adolescents weighing under 40Kg because of the need for at least $2x10^7$ leukocytes per Kg. for better transplantation (Kurtzberg et al. 1996; Wagner et al. 1996; Kapelushnik et al. 1998; Shpall et al. 2000; Jaroscak et al. 2003a). On the other hand, more purified populations affect the success of

transplantation (Bensinger et al. 1996a; Negrin et al. 2000; Richel et al. 2000; Laughlin et al. 2001). The facts indicated above clearly demonstrate why highly sophisticated cultivation techniques and bioreactor concepts are needed to improve survival and efficacy of cellular therapy applications. Furthermore, in order to use cells in clinical trials and as biopharmaceutical product, there is a need to control the culturing conditions as much as possible. Such conditions can be only obtained in different types of bioreactors in which the cells are protected from the external surrounding and as much of their conditions are controlled.

Parameters for Cultivation of Stem Progenitor Cells in Bioreactors

The intricate regulation governing the growth and differentiation of HSC, MSC or EPC is controlled both by the cellular microenvironment (epigenetic signals and development) as well as genetic factors (genetic development). When cultivating cells in vitro, it is essential to carefully consider the importance of chemical and physical variables such as composition of growth media, oxygen concentration, pH levels, and osmolarity, as well as the specific design and operation of the vessel in which the culture is to be maintained. It is even further important when attempting to culture cells, such as stem and progenitor cells that are at the beginning of their ontogenetic development, as these cells can be very sensitive to many signals such as paracrine and autocrine signals, contact signals and levels of Oxygen, carbonate, glucose and other nutrients.

In the following we summarize key issues to be considered while utilizing bioreactors and scaffolds in combination with Gamida Cell's technologies for ex vivo expansion of stem and or progenitor cells with lesser extent of differentiation. These culturing techniques are of the static, stirred and immobilized as previously explained.

Bioreactors.

A bioreactor is a generalizing name that essentially covers any kind of vessel that is capable of incubating cells while protecting the cells' environment at least at some level. It can be a static vessel such as a flask or culture bag in which the variables (such as composition of growth media, oxygen concentration, pH levels, and osmolarity) are not fully controlled and detected. On the other hand stand the fully automated electromechanical state of the art bioreactors in which all the variables are

controllable and detected. Many inter-combinations between those extremes are commonly used in cellular biotechnology.

Three different traditional approaches for the cultivation of isolated hematopoietic stem or progenitor cells have been described in the literature, the static, the stirred and the immobilized culture. Static cultivation takes place in very simple culture systems like well plates, tissue-culture flasks or gas-permeable culture bags (Brugger et al. 1995; Alcorn et al. 1996). As the first two systems do not allow cell cultivation on a clinical scale, the latter is actually the most often used technique for stem cell expansion (Purdy et al. 1995; McNiece et al. 1999; McNiece et al. 2000a). All these systems have the advantage of being easy to handle, single-use devices, which enable an uncomplicated cell harvest. But with all of them the possibility for process control is only what can be maintained in the incubator and there is no possible of continuous feeding. This causes variations in culture conditions during cultivation (e.g., oxygen tension, pH, substrate, metabolite and cytokine concentrations).

Stirred bioreactors are common in animal cell culture, as they offer a homogenous environment, representative sampling, better access to process control and an increased oxygen transfer. Several of these techniques (spinner flasks and stirred vessel bioreactors) have been tested successfully for the cultivation of hematopoietic cells (Zandstra et al. 1994; Collins et al. 1998a; Collins et al. 1998b; Noll et al. 2002).

The immobilization of stem and progenitor cells is an attempt to reach local high cell densities and to imitate the three-dimensional structure of the bone marrow without the use of stroma. A number of porous microcarriers with and without additional coating of components of an extra-cellular matrix hydrogel (e.g., collagen, fibronectin, laminin) have been investigated for this purpose. Bagley et al. compared different porous materials and described a greater than sixfold expansion of colony forming cells in a long-term cultivation of CD34+ cells in tantalum-coated porous carriers, even without adding exogenous cytokines (Bagley et al. 1999). However, stem cell immobilization, especially in porous materials, requires a technique and time for detaching the cells from the matrix prior to transplantation, a significant disadvantage compared to suspension culture.

Hollow fiber modules and the micro-encapsulation of progenitor cells have been used in hematopoietic culture with less success (Sardonini and Wu 1993). Furthermore, these approaches do not fit the clinical requirements, as the harvest of the cells is almost always impossible.

The most ambitious technique for stem cell expansion to date is the Aastrom-Replicell system (Aastrom Biosciences Inc., Ann Arbor, MI, USA), which is an automated clinical system for the onsite expansion of stem cells in cancer therapy.

It consists of a grooved perfusion chamber for the retention of the hematopoietic cells, with the medium flow perpendicular to the channel grooves resulting in a continuous supply of fresh nutrients while metabolites are simultaneously removed (Sandstrom et al. 1995; Koller et al. 1998). This technique has already been used in a number of clinical studies (Chabannon et al. 1999a; Chabannon et al. 1999b).No incompatibility of the expanded cells was found, but the expansion of the early progenitor cells was rather low (Chabannon et al. 1999a; Jaroscak et al. 2003a).

Local high cell densities, as they are realized in the pores of microcarriers or in the grooves of the Aastrom Replicell, have been described to make bone marrow MNC essentially stroma-independent in terms of long term cell maintenance and expansion (Koller et al. 1998). This might also be the reason for the good expansion of progenitors in the culture bags, where the cells accumulate in the wrinkles of the bag and reach local high cell densities (Purdy et al. 1995; McNiece et al. 1999; McNiece et al. 2000a).

The following is a summary of the different bioreactors and hydrogel scaffolds that can be utilized in relation to this invention.

Static Bioreactors: Culture VueLife® FEP Teflon bags (American Fluoroseal Corporation, Gaithersburg, MD) are a good example of the static bioreactor. The cells are cultured within the culture bag that is incubated in an incubator that keeps its temperature, humidity, CO_2 levels and other essential parameters. This very basic bioreactor was used many time for large production of hematopoietic stem cells (Briddell et al. 1997; LaIuppa et al. 1997; McNiece et al. 2000b).

Stirred Bioreactors (spinner flask): More advanced dynamic electromechanical bioreactors have additional features. The spinner flask (bottle) is the basic fluid-dynamic cultivation vessel we use. It is an agitated flask (bottle)

usually running at 50 rpm (Carrier et al. 1999). The cell constructs (or suspension), in these vessels are subjected to turbulent mixed media that provides a well-mixed environment around the cells and thus minimize the stagnant layer at their surface. These spinner flasks are commonly maintained in an incubator that provides their essential physical conditions (Carrier et al. 1999).

*Stirred Bioreactors (Perfused bioreactors):*In addition to spinner flasks more advanced perfusion bioreactors would be utilized. Basically the "perfusion bioreactors" can be classified roughly into two groups based on their feeding procedure. While one type is fed continuously the other is fed in pulses.

Bioreactor Materials. Sensitivity to constructing material is unrelated to whether cells are anchorage-dependent or not, with material upkeep (sterilization, cleaning, and multiple using) significantly affecting culture survival (LaIuppa et al. 1997). This indicates that rather than an essential role for cell-surface interactions, bioreactor materials may affect the culture by percolating toxins or binding essential media factors. This was highlighted by the finding that a small silicon seal inside the agitator shaft of a spinner flask may impair the ability of the culture to grow in suspension (Sardonini and Wu 1993; Zandstra et al. 1994).

Scaffold Bioreactors. Recent attention has focused on the culturing of dissociated tissues in three-dimensional polymeric scaffolds, providing the initial support required for tissue organization and matrix construction. The building matter for such as scaffold would ideally be of sponge-like, biodegradable, porous composition, and support future clinical uses such as transplantation. To support this function, the scaffold has to be able to support vascularization. The morphology of the scaffold can vary greatly according to pore size, pore wall thickness, elasticity, and building material. Such scaffolds have been described as able to support cardiomyocyte viability (Dar et al. 2002), as well as hematopoietic progenitor cell survival (Banu et al. 2001).

Hydrogel scaffolds: For cells to survive well and proliferate within a scaffold an aqueous environment should be maintained. Such an environment is achieved in polymers (mainly polypeptides, polysaccharides or a combination of the two) that form hydrogels. Several types of hydrogels that portray different characteristics such as porosity, cell-hydrogel interactions and degradation properties are used in this study. The hydrogels and other scaffolds may be used for: A. to support 3D structures

within the bioreactors and by that providing the cells with better expanding conditions. B. As an encapsulated survival environment for cells to be transplanted in vivo into animal models and later into patients. In the latter scenario the scaffold can be regarded as a bioreactor or the incubating vehicle within the bioreactor (the whole animal). Roughly, the scaffolds used for ex vivo expansion of cells and for tissue engineering can be divided into two groups: Synthetic polymers and Natural polymers. Synthetic polymers provide a precise control of physical properties such as molecular weight, 3D structure, degradation time, mechanical strength and flexibility and hydrophobicity. As said the invention can utilize any given hydrogel as long it is suitable for the survival of cells. Among these hydrogels are many types of Synthetic polymers like Polyhydroxyalkanoate, poly 4 hydroxybutirate (P4HB), polygluconic acid (PGA), poly lactate (PLA) and other combinations of these polyesters or other synthetic hydrogels. Other hydrogels, the natural occurring polymers, mainly fibrin based, alginate, collagen, fibronectin, agarose, pullulan, dextran sulfate, hyaluronic acid and other natural hydrogels. The different hydrogels for applications of production in bioreactors and other regenerative medicine applications are excellently summarized in tables 1A and 1B page 63 in Hoffman 2001.

Growth Media Ingredients: In the bone marrow, cytokines are produced predominantly from stromal cells (Linenberger et al. 1995; Lisovsky et al. 1996; Guerriero et al. 1997), although accessory and hematopoietic cells themselves have also been shown to secrete growth factors (even such as Stem Cell Factor SCF, Linenberger et al. 1995). Cytokines affect chiefly all processes of hematopoiesis, like proliferation, differentiation, adhesion and functionalities of the cells, while, in the absence of cytokines, HSC probably suffer from programmed cell death, apoptosis (Cotter et al. 1994). The effects of hematopoietic cytokines are very complex and show both synergistic as well as antagonistic interactions. Changes in the cytokine concentrations during cultivation can cause significant changes in the proliferation and the differentiation of the cells. Therefore, the control of cytokine composition is an extremely important element of the bioprocess strategy. For the expansion of stem and progenitor cells, interleukin 6 (IL-6), stem cell factor (SCF), thrombopoietin (TPO) and flt3 ligand (FLt3) are thought to play major roles and are mostly used in the expansion of hematopoietic stem and progenitor cells (Piacibello et al. 1997; Murray et al. 1999; Ramsfjell et al. 1999).

The number of cytokines known to influence hematopoiesis is steadily growing but there are still growth factors in the stromal environment to be identified. This has been proven by the additional growth-supportive effects of stroma-conditioned medium on the proliferation of hematopoietic stem cells.

The choice of the culture medium, especially the use of serum, directly influences the differentiation of the cells and therefore the aims of cultivating HSC, MSC or EPC should be considered when determining the medium to be used (McAdams et al. 1996a). For example, serum normally contains TGF-*b*, which is known to inhibit the erythroid and megakaryocytic lineage, therefore promoting the granulocytic and macrophage differentiation (Dybedal and Jacobsen 1995). In stroma-containing culture, serum strengthens the adhesion of the cells and stabilizes the feeder layer. A further aspect which has to be considered in the use of animal serum (e.g., fetal bovine or horse) is the clinical applicability, as media containing components from animal sera will face more regulatory hurdles than serum-free compositions (Sandstrom et al. 1996).

Because hematopoiesis in the bone marrow takes place under static conditions (McAdams et al. 1996a), with a continuous feed of nutrients and a simultaneous removal of waste products, several feeding strategies have been developed in the cultivation of hematopoietic cells.

Growth Media Feeding. Various methods have been employed for feeding cultures, ranging from feeding of cells cultured in culture bags once weekly or even less (McNiece et al. 2000b; McNiece and Briddell 2001; McNiece 2001) to half-medium exchange per week (in one feeding paradigm), to complete daily medium exchange in another scheme (Schwartz et al. 1991). Although continual feeding of fresh medium is theoretically beneficial for removal of waste products that may be growth-inhibitory (e.g., lactate, Patel et al. 2000), it could potentially eliminate key autocrine signals that may be important for self-regulating expansion signals emitted by the stem cell population, as well as prove costly if feeding relies on a continual supply of a costly additive.

Oxygen Tension. The role of oxygen tension in best illustrated in the direction of hematopoietic differentiation (McAdams et al. 1996a; McAdams et al. 1996b). Low oxygen concentrations promote proliferation of colony-forming cells, perhaps by augmenting the effects of growth factors such as Epo while lessening oxidative

damage. Modulation of oxygen levels thus pose a serious challenge to cell culture efforts due to the combined necessity of accurate measurement as well as oxygen flow control. Low oxygen concentration (hypoxia) was recently found to favor renewal and proliferation of hematopoietic stem cells (Danet et al. 2003)

pH. Local pH conditions vary with respect to different cell lineages. Low pH levels (<6.7) do not allow any hematopoietic proliferation, with erythroid differentiation specifically requiring a minimal level of pH 7.1. Optimal pH levels were found to be 7.2-7.4 for proliferation of GM-CSF, and 7.6 for erythroid cells (McAdams et al. 1996a). The pH can have a further impact on growth and proliferation of stem/progenitor cells as it is corresponding closely to internal calcium concentrations that are essential for proper development

Osmolality. An optimal range for culturing of MNC and CD34+ cells was recently described between 0.31 and 0.32 mOsmol/kg (Noll et al. 2002). The CD34+ population shows extreme sensitivity to osmolality (beyond the linear effects seen on the MNC). In addition, Osmolality, like pH, can be an efficient modulator of lineage-specific differentiation, as progenitors of granulocytic and macrophages peak at hypotonic osmolalities (0.29 mOsmol/kg), while BFU-E proliferation is enhanced at hypertonic levels (0.34 mOsmol/kg).

Shear and Mixing Effects. Due to their anchorage-independent characteristics, hematopoietic cells of all lineages have been shown to grow exceedingly well in stirred culture systems (unlike fibroblasts and endothelial cells), potentially due to the elimination of diffusion and gradient limitations imposed by the static systems. In contrast, cellular sensitivity to shearing is most pronounced for hematopoietic cells, being able to withstand only about 30 revolutions per minute (Collins et al. 1998a). In addition, it is an accepted fact that stirring modulates the physical and metabolic characteristics of the culture, as changes in surface marker expression has been observed with such cultures (McDowell and Papoutsakis 1998). Interestingly, this provides yet an additional method for controlling the cell fate of cultures.

Since we are interested in methodologies for large-scale production of stem/progenitor cells with lesser extent of differentiation, we initiated several studies that scrutinize the feasibility and the yields of combining Gamida Cell's ex vivo expansion technologies with technologies for large-scale production in bioreactors. In

this invention we demonstrate the novel combination of Gamida Cell's technologies to induce ex vivo expansion of stem/progenitor cells with bioreactor technologies to yield large quantities of less differentiated HSC, MSC or EPC. In a further embodiment of the invention we demonstrate that the ex vivo expansion of these cells with Gamida Cell's technologies may happen while incubated in a hydrogel scaffold either while in any kind of a bioreactor or when incubated in the patient or the animal model.

Gamida Cells technologies to induce ex vivo expansion of stem or progenitor cells. In this invention we utilize Gamida Cell's unique technologies to induce ex vivo expansion of stem/progenitor cells with bioreactor technologies to yield larger quantities of less differentiated HSC, MSC or EPC. In brief the technologies involve copper chelation that allow proliferation while reducing the extent of differentiation of the stem/progenitor cells involved (Peled et al. 2002 and patents # WO0018885A1, WO9940783A1, US20020114789A1, US20020159981A1). Gamida Cell technologies are summarized in Patents. Additional techniques reduce the expression and/or activity of CD38 (an membrane-bound NAD+ glycohydrolases enzyme involved in ADP-ribosyl cyclases synthesis) as explained in a continuation in part of U.S. Patent Application No. 09/986,897 (Peled T., EXPANSION OF RENEWABLE STEM CELL POPULATIONS).

Results

We will first show the synergistic nature of combining Gamida Cell's technologies with bioreactor technologies by demonstrating GC technologies in a static bioreactor, in our case, the Teflon gas permeable culture bags. As an example we will present ex vivo expansion of hematopoietic stem cells but the data for MSC or EPC is very similar. As copper chelation is the current leading technology of Gamida Cell (Peled et al. 2002) it's utilization with the different blood derived stem cells in different types of bioreactors would be evaluated first. The following experiment compares the outcome of ex vivo expansion of HSC in gas permeable culture bags with GC technology of copper chelation to those with Gamida Cell's technology.

Copper chelation and ex vivo expansion of HSC in a gas permeable culture bag. Mononuclear cells (MNC) were collected from either bone marrow (BM), mobilized peripheral blood (MPB) or umbilical cord blood (UCB, as in figure

1) and Hematopoietic stem/progenitor cells are isolated by magnetic activated cell sorting (MACS technology, Milteny, Bergisch-Gladbach, GmbH) as explained in M&M. The HSC are then seeded in gas permeable culture bags at concentrations of $1x10^4$ cells/ml in MEM-alpha with 10% Fetal Calf Serum (FCS) containing 50 ng/ml of the following cytokines: SCF, TPO, Flt-3, IL-6 and incubated for at least three weeks in a 5%CO_2 humidified incubator. The culture bags are divided to two groups while the first is supplemented with 5μM of GC's leading copper chelator tetraethylenepentamine (TEPA, Aldrich, Milwaukee WI, USA) the other group is not. The culture bags are then fed once weekly with the same media components. Figure 1A and B shows the fold expansion of subpopulations of HSC following three weeks of such culture. The two subpopulations $CD34^+/CD38^-$ and $CD34^+/lin^-$ are considered to represent the immature subpopulation of HSC, i.e., the subpopulation that has the major role in self-renewal and proliferation of the HSC. As can be concluded from Fig. 1A and 1B incubation of the cells in the static bioreactor with 5μM TEPA dramatically increases the fold expansion of these immature subpopulation, indicating the higher long-term potential of this treatment. Furthermore, Fig 1C shows that in a functional assay, Long Term Culture-Colony Forming Cell (LTC-CFC assay) co-incubation of HSC with 5μM TEPA increase their numbers by about one order of magnitude as compared to control (non-treated) cells. We will study further the synergism between GC different technologies (see M&M) and the static vessel and we will show them not only for HSC but also for MSC and EPCs.

Consequently, we will show that the outcome of the combinations of GC technologies and the static bioreactor (culture bag) is further enhanced when the cells are cultivated within a hydrogel scaffold that was shown to have favorable conditions. We will study several hydrogels to find the best candidate for each type of stem or progenitor cells. Among the hydrogel to test would be alginate, fibronectin, laminin, collagen, hyaluronic acid, Polyhydroxyalkanoate, poly 4 hydroxybutirate (P4HB) and polygluconic acid (PGA). See the materials and methods for partial list of hydrogels we want to test. We will test them not only for HSC but also for MSC and EPCs. In a further development of this issue we will transplant cells within the scaffolds into animal models and test their ability to serve as in vivo bioreactors.

Another branch of the study will deal with non-static bioreactors:

We will first use spinner flasks and upon optimization we will show that this technology is synergistic to the said GC technologies. We will validate the proper media for the highest possible expansion of the stem cells and determine the conditions that will enable the expansion of these cells without animal sera. We will develop further our findings with spinner flasks (or bottles) that utilize hydrogels as scaffolds that we found to be beneficial.

Subsequently, we will use state of the art fully automated continuous-flow perfusion bioreactors that control almost every possible variable. We will look at each controllable variable and will see its effect on the ex vivo expansion of the stem cells and the effect of GC different technologies. We will validate the proper media for the highest possible expansion of the stem cells and determine the conditions that will enable the expansion of these cells without animal sera. We would like to show that the said technologies are synergistic and that the amount of fold expansion is higher with them. We will develop further our findings to utilize hydrogels as scaffolds for growth of the cells in such sophisticated perfusion bioreactor and will evaluate these scaffolds' abilities to increase the number of cells. Ultimately, we may want to study the possibility of utilizing the said technologies in 3D tissue culturing, especially with MSC that are known to differentiate to so many types of tissues (Pittenger et al. 1999).

Materials and Methods

Cell source

The cells in the present invention would be from one of the following sources:

1. Hematopoietic stem cells (HSC) or progenitor cells (HPC) from either bone marrow (BM), G-CSF mobilized peripheral blood (MPB) or umbilical cord blood (UCB)
2. Human Mesenchymal stem cells (hMSC) from either bone marrow (BM), G-CSF mobilized peripheral blood (MPB) or umbilical cord blood (UCB).
3. Endothelial Progenitor Cells (EPC, Rafii and Lyden 2003) from either bone marrow (BM), G-CSF mobilized peripheral blood (MPB) or umbilical cord blood (UCB).

Cell cultures of HSC or HPC. Cells will be obtained from UCB after a normal full-term delivery (informed consent was given), from PB, or BM donations.

UCB Samples will be collected and processed within 12 hours postpartum. Blood will be mixed with 3% Gelatin (Sigma, St. Louis, MO) and be sedimented for 30 minutes to remove most RBC. The leukocyte-rich fraction will be harvested and layered on Ficoll-Hypaque gradient (1.077 g/mL; Sigma), and centrifuged at 400 g for 30 minutes. The mononuclear cells in the interface layer will be then collected, washed three times, and resuspended in phosphate-buffered saline (PBS) (Biological Industries) containing 0.5% bovine serum albumin (BSA) (Fraction V; Sigma). To purify the $CD34^+$ or $CD133^+$ cells, the mononuclear cell fraction will be subjected to two cycles of immuno-magnetic separation using the "MiniMACS CD34 or CD133 progenitor cell isolation kit" (Miltenyi Biotec, Bergisch-Gladbach, GmbH), accordingly, following the manufacturer's recommendations. Purified CD34+ or $CD133^+$ cells will be cultured in non tissue culture treated culture flasks at a concentration of $1x10^4$ cells/ml in either MEMα/10% FCS (Biological Industries) containing the following human recombinant cytokines: Thrombopoietin (TPO), interleukin-6 (IL-6), FLT-3 ligand and stem cell factor (SCF), each at a final concentration of 50 ng/ml (PeproTech, Inc., Rocky Hill, NJ, USA) or serum-free medium (such as StemSpan; Stem Cell Technologies Inc., Vancouver, British Columbia, Canada, or X-VIVO 15 or 20, BioWhittaker Inc. Walkersville, MD or any other cGMP grade serum free media commercially available) containing 300 ng/ml of stem cell factor (SCF), 100 ng/ml of Thrombopoietin (TPO), 300 ng/ml of flt-3 ligand (FL), 100 ng/ml of IL-6, 20 μg/ml of LDL (Sigma-Aldrich). All cultures are incubated at 37°C in a humidified atmosphere of 5% CO_2 in air before utilization with the different bioreactors.

Mesenchymal stem cells isolation and culture. Mesenchymal stem cell (MSC) cultures will be prepared as previous described by Pittenger et al. 1999 and Benayahu et al. (Shur et al. 2001; Shur et al. 2002). Cells collected from surgical aspirates of bone marrow, UCB or PB to prepare *ex vivo* culture will be plated at low-density ($1.5x10^4$ cells/cm^2) and cultured in growth medium containing Dulbecco's Modified Essential Medium (DMEM) with the addition of 10% heat-inactivated fetal calf serum (FCS) (Biological industries, Bet-Haemek, Israel). To generate large number of cells from the primary cultures, the cells will be trypsinized and single cell suspensions will be re-cultured for 7 days and grown up to 80% confluence and incubated at 37°C humidified atmosphere with 5%CO_2 for 3 days before the first medium change. The mesenchymal

population will be isolated based on its ability to adhere to the culture plate (Wakitani et al. 1995; Pereira et al. 1998; Sakai et al. 1999). Following the first medium change, subsequent changes will be done twice a week. At 90% confluence, the cells will be trypsinized (0.25% Trypsin-EDTA, Sigma) and will be passaged to 225 cm^2 flasks at 1:3 ratios. These first passage MSCs will be used in all experiments.

In order to assess the percentage of MSCs out of the total cells that will be transfused, we will use the polyclonal antibody to the MSCs surface antigen SB-10 (ALCAM) (Santa Cruz Biotechnology, Bruder et al. 1998).

Preparation of endothelial progenitor cells. BM, MPB and UCB derived endothelial progenitor cells (EPCs) will be prepared as described elsewhere (Kawamoto et al. 2003). CD31 (+) cells will be separated by a Miltenyi Biotec's magnetic cell separation technology (MACS) and suspended in X vivo-15 medium (Biowhittaker) supplemented with 1 ng/mL carrier-free human recombinant VEGF (R&D), 0.1 μmol/L atorvastatin (Pfizer), and 20% human serum drawn from each individual patient. Cells will be seeded at a density of $6.4x10^5$ cells/mm2 at fibronectin-coated dishes (Roche). After 3 days of cultivation, cells will be detached with 0.5 mmol/L EDTA, will be washed twice and resuspended in a final volume of 10 mL X vivo-10 medium. The resulting cell suspension contains a heterogeneous population of progenitor cells.

Technologies to induce ex vivo expansion of stem or progenitor cells.

Copper chelation Mainly 5-10 μM of the leading copper chelator tetraethylenepentamine (TEPA, -Aldrich, Milwaukee WI, USA) will be used according to cell type and bioreactor.

Reduction of the expression and/or activity of CD38 By compounds and techniques as explained in a continuation in part of U.S. Patent Application No. 09/986,897 (Peled T., EXPANSION OF RENEWABLE STEM CELL POPULATIONS).

The polyamine chelators and regulators of expression and/or activity of CD38 would be added to the culturing media within the feeding cycles. The indicated concentration of these low molecular weight compounds would be as presented by Gamida Cell in the different patent invention summaries covering these techniques for each type of cells. In general, molecules like TEPA would be utilized at the range of

2.5-25 μM (i.e., at concentrations within the same order of magnitude of their reported effect). The same formulation would be utilized for other molecules invented by Gamida Cell in order to ex vivo expand stem and progenitor cells.

Bioreactors.

Culture Teflon Bags. VueLife® FEP Teflon bags (American Fluoroseal Corporation, Gaithersburg, MD) will be used at volumes of 72 or 290 ml. Other gas permeable tissue culture bags (either made of FEP or other material/s can be easily used as well). When the Teflon bag is utilized the cells are incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Mechano-electrical bioreactors. Several types of bioreactors that portray different patterns of fluid dynamics and vessel geometry to improve mass transport are used in this study. The mechano-electrical bioreactors can be anyone of the bioreactors previously used for the cultivation of HSC, MSC or EPC or other stem cells that are described in the scientific literature (Koller et al. 1993a; Koller et al. 1993b; Zandstra et al. 1994; Koller et al. 1995; Collins et al. 1998a; Collins et al. 1998b; Kogler et al. 1998; Mantalaris et al. 1998; Chabannon et al. 1999a; Nielsen 1999; Leor et al. 2000; Banu et al. 2001; Mackin et al. 2001; Altman et al. 2002; Dar et al. 2002; Mandalam and Smith 2002; Noll et al. 2002; Sen et al. 2002a; Sen et al. 2002b; Wolff 2002; Jaroscak et al. 2003b) or are used in patents (such as in patents: US5728581, US5605822, WO9640876A1, WO02064755A2, US 5811301, WO9514078A1, WO02080995A1, WO974707, WO0046349, WO03004626A1,A2,A3, US6228635, WO0066712A3, WO0066712A2, US5985653, US5928945,US5843431, US5833979, US5824304, US5795790, US5776747, US5645043, US5635387, US5635386, WO9521911A1, US5646043, US5437994, US5605822, US5635386, US5646043, US5670351, US6326198, US5674750, US5925567).

We will briefly present common mechano-electrical bioreactors: The spinner flask (bottle) is the basic fluid-dynamic cultivation vessel we use. It is an agitated flask (bottle) usually running at 50 rpm (Carrier et al. 1999). The cell constructs (or suspension), in these vessels are subjected to turbulent mixed media that provides a well-mixed environment around the cells and thus minimize the stagnant layer at their surface.

In addition to spinner flasks more advanced perfusion bioreactors would be utilized. Basically the "perfusion bioreactors" can be classified roughly into two groups based on their feeding procedure. While one type is fed continuously the other is fed in pulses.

As examples of such perfusion bioreactors we will further present OPTICELL™ OPTICORE™ ceramic core S-51, S451 (flat surface area 23.8m^2), S-1251 (flat surface area 10.4m^2) or S- 7251 (Cellex Biosciences, Inc., Minneapolis; Minn.). These bioreactors are initially sterilely perfused, preferably for 1-3 days, with sterile deionized water to remove any toxic substances adhering to the core. Thereafter, the core is perfused for a brief period (less than 24 hours) with sterile 25% (w/v) human serum albumin in order to coat the core with protein. The bioreactor core is next perfused for 4-24 hours with a sterile solution of an anticoagulant, preferably heparin sulfate, 100 U/mL 65 (Upjohn Co.) as a source of glycosaminoglycan and to prevent cell clumping during HSC inoculation. Following this preparation, the core is conditioned by perfusing it with sterile human HSC medium (see **Cell culture of HSC** above), preferably for about 24 hours, prior to inoculating the bioreactor with HSC.

Bioreactor Culture System

The culture system consists of a variable number of bioreactors connected to the medium source by sterile plastic tubing. The medium is circulated through the bioreactor with the aid of a roller or centrifugal pump (e.g., KOBETM) or a peristaltic pump. Probes to measure pH, pO_2 and pCO_2 as well as shear stress and temperature are located in line at points immediately before and following the bioreactor(s). Information from these sensors is monitored electronically. In addition, there is a mean of obtaining serial samples of the growth medium in order to monitor glucose, electrolytes, cytokines and growth factors and nutrient concentrations. Activities of cytokines and growth factors are measured by conventional bioassays (e.g., colony forming assays or dependent cell line growth assays) or conventional immunoassays.

Large scale production of hematopoietic Stem Cells, MSC or EPCs.

A number of HSCs/MSCs/EPCs appropriate to the size of the bioreactor, at a concentration of about 1x10^5-2x10^7 cells/mL, are mixed with an equal volume of serum containing/free media and injected into the bioreactor. In case of MSCs/EPCs circulation of the growth medium is interrupted for a period of about 1-4 hours in

order to permit the cells to attach to the surface of the bioreactor core or capillaries with HSC there is no need for such a protocol. Thereafter, the circulator pump is engaged and the growth medium pumped through the system at an initial rate determined by the size of the reactor; a typical rate is about 24 mL/min. Gas exchange occurs via silicone tubes (surface area=0.5 m^2) within a stainless steel shell, or by a conventional membrane oxygenator. Polarographic O_2 and CO_2 probes and autoclavable pH electrodes monitor O_2 and CO_2 tensions and pH continuously, respectively. Flow rates are adjusted so as to maintain an optimal O_2 tension (a partial pressure of at least about 30-50mm of Hg) and optimal physiologic pH (7.30-7.45). Low oxygen concentration (hypoxia) was recently found to favor renewal and proliferation of hematopoietic stem cells (Danet et al. 2003).

When an appropriate number of cells are obtained, as determined by oxygen utilization of the system, a second bioreactor may be connected to the system, and cells fed directly into this second bioreactor. Thereafter, the second bioreactor is flushed with fresh growth medium and maintained for up-to 5 weeks for cultivation of the desired hematopoietic components.

Scaffolds and Hydrogels.

For cells to survive well and proliferate within a scaffold an aqueous environment should be maintained. Such an environment is achieved in polymers (mainly polypeptides, polysaccharides or a combination of the two) that form hydrogels. Several types of hydrogels that portray different characteristics such as porosity, cell-hydrogel interactions and degradation properties are used in this study. The hydrogels and other scaffolds may be used for: A. to support 3D structures within the bioreactors and by that providing the cells with better expanding conditions. B. As an encapsulated survival environment for cells to be transplanted in vivo into animal models and later into patients. In the latter scenario the scaffold can be regarded as a bioreactor or the incubating vehicle within the bioreactor (the whole animal). Roughly, the scaffolds used for ex vivo expansion of cells and for tissue engineering can be divided into two groups: Synthetic polymers and Natural polymers. Synthetic polymers provide a precise control of physical properties such as molecular weight, 3D structure, degradation time, mechanical strength and flexibility and hydrophobicity. As said the invention can utilize any given hydrogel as long it is suitable for the survival of cells.

Among these hydrogels are many types of Synthetic polymers like Polyhydroxyalkanoate, poly 4 hydroxybutirate (P4HB), polygluconic acid (PGA), poly lactate (PLA) and other combinations of these polyesters or other synthetic hydrogels that are summarized in table 1B page 63 in Hoffman 2001. These hydrogels are reported in scientific literature (e.g., Kogler et al. 1998; Robinson et al. 1999; Williams et al. 1999; Sodian et al. 2000; Cascone et al. 2001; Hoffman 2001; Low et al. 2001; Papadaki 2001; Christensen et al. 2002; Griffith and Naughton 2002; Noll et al. 2002; Risbud and Sittinger 2002; Sen et al. 2002a; Sodian et al. 2002; Abukawa et al. 2003) or in Patents (e.g., US6585994, US6555123, US20030072784A1, US6514515, US66495152, WO0119422A1, WO0216627A2,A3, WO0119361A2,A3, US6129761, US6040493, WO02081662A1, US6179862, US6165201, US5964745, US5855610, US5741685, US5709854). Other hydrogels, the natural occurring polymers, mainly fibrin based, alginate, collagen, fibronectin, agarose, pullulan, dextran sulfate, hyaluronic acid and other natural hydrogels are summarized in table 1A page 63 in Hoffman 2001. These hydrogels are reported in scientific literature (e.g., Shapiro and Cohen 1997; Tock et al. 1998; Robinson et al. 1999; Cohen et al. 2000; Leor et al. 2000; Cassell et al. 2001; Currie et al. 2001; Hildebrand et al. 2001; Hoffman 2001; Robinson et al. 2001; Arts et al. 2002; Dar et al. 2002; Geer et al. 2002; Grassl et al. 2002; Haisch et al. 2002; Huang et al. 2002; Manosroi et al. 2002; Spitzer et al. 2002; Wechselberger et al. 2002; Borges et al. 2003; Groger et al. 2003; Hojo et al. 2003; Karp et al. 2003; Neovius and Kratz 2003) or in Patents (e.g., US6461325, US6074663, US6548729, WO02089868A1, WO02070795A1, WO0185225A2,A3, WO9915209A2, US6506365, EP1061931A2, US5716645, WO9525748A1, US6281341, US5976780, US5578314, US6451060, US6423312, US6264701, US6197061, US5981825, US6425918, US6334968, US5876742, US5709854, US6365385, US6348069, US5855610, WO02080995A1, WO0239948A2,A3, WO0102030A2,A3, US6517872, US20030078672A1, US6586011, US6425918, US6334968, WO9965463A1, EP0901384A1, WO9744070A1).

References

Abukawa, H., H. Terai, D. Hannouche, J. P. Vacanti, L. B. Kaban and M. J. Troulis (2003). "Formation of a mandibular condyle in vitro by tissue engineering." J Oral Maxillofac Surg **61**(1): 94-100.

Alcorn, M. J., T. L. Holyoake, L. Richmond, C. Pearson, E. Farrell, B. Kyle, D. J. Dunlop, E. Fitzsimons, W. P. Steward, I. B. Pragnell and I. M. Franklin (1996). "CD34-positive cells isolated from cryopreserved peripheral-blood progenitor cells can be expanded ex vivo and used for transplantation with little or no toxicity." J Clin Oncol 14(6): 1839-47.

Altman, G. H., H. H. Lu, R. L. Horan, T. Calabro, D. Ryder, D. L. Kaplan, P. Stark, I. Martin, J. C. Richmond and G. Vunjak-Novakovic (2002). "Advanced bioreactor with controlled application of multi-dimensional strain for tissue engineering." J Biomech Eng **124**(6): 742-9.

Andrews, R. G., R. A. Briddell, F. R. Appelbaum and I. K. McNiece (1994). "Stimulation of hematopoiesis in vivo by stem cell factor." Curr.Opin.Hematol. 1(3): 187-196.

Arts, C. H., P. P. Hedeman Joosten, J. D. Blankensteijn, F. J. Staal, P. Y. Ng, G. J. Heijnen-Snyder, J. J. Sixma, H. J. Verhagen, P. G. de Groot and B. C. Eikelboom (2002). "Contaminants from the transplant contribute to intimal hyperplasia associated with microvascular endothelial cell seeding." Eur J Vasc Endovasc Surg **23**(1): 29-38.

Bachier, C. R., E. Gokmen, J. Teale, S. Lanzkron, C. Childs, W. Franklin, E. Shpall, J. Douville, S. Weber, T. Muller, D. Armstrong and C. F. LeMaistre (1999). "Ex-vivo expansion of bone marrow progenitor cells for hematopoietic reconstitution following high-dose chemotherapy for breast cancer." Exp.Hematol. **27**(4): 615-623.

Bagley, J., M. Rosenzweig, D. F. Marks and M. J. Pykett (1999). "Extended culture of multipotent hematopoietic progenitors without cytokine augmentation in a novel three-dimensional device." Exp Hematol **27**(3): 496-504.

Banu, N., M. Rosenzweig, H. Kim, J. Bagley and M. Pykett (2001). "Cytokine-augmented culture of haematopoietic progenitor cells in a novel three-dimensional cell growth matrix." Cytokine **13**(6): 349-58.

Barker, J. N. and J. E. Wagner (2002). "Umbilical cord blood transplantation: current state of the art." Curr.Opin.Oncol. 14(2): 160-164.

Bensinger, W. I., C. D. Buckner, K. Shannon-Dorcy, S. Rowley, F. R. Appelbaum, M. Benyunes, R. Clift, P. Martin, T. Demirer, R. Storb, M. Lee and G. Schiller (1996a). "Transplantation of allogeneic CD34+ peripheral blood stem cells in patients with advanced hematologic malignancy." Blood 88(11): 4132-4138.

Bensinger, W. I., S. D. Rowley, T. Demirer, K. Lilleby, K. Schiffman, R. A. Clift, F. R. Appelbaum, A. Fefer, T. Barnett, R. Storb, T. Chauncey, R. T. Maziarz, J. Klarnet, P. McSweeney, L. Holmberg, D. G. Maloney, C. H. Weaver and C. D. Buckner (1996b). "High-dose therapy followed by autologous hematopoietic stem-cell infusion for patients with multiple myeloma." J.Clin.Oncol. 14(5): 1447-1456.

Borges, J., F. T. Tegtmeier, N. T. Padron, M. C. Mueller, E. M. Lang and G. B. Stark (2003). "Chorioallantoic membrane angiogenesis model for tissue engineering: a new twist on a classic model." Tissue Eng 9(3): 441-50.

Briddell, R. A., B. P. Kern, K. L. Zilm, G. B. Stoney and I. K. McNiece (1997). "Purification of CD34+ cells is essential for optimal ex vivo expansion of umbilical cord blood cells." J.Hematother. 6(2): 145-150.

Bruder, S. P., N. Jaiswal, N. S. Ricalton, J. D. Mosca, K. H. Kraus and S. Kadiyala (1998). "Mesenchymal stem cells in osteobiology and applied bone regeneration." Clin Orthop(355 Suppl): S247-56.

Brugger, W., S. Heimfeld, R. J. Berenson, R. Mertelsmann and L. Kanz (1995). "Reconstitution of hematopoiesis after high-dose chemotherapy by autologous progenitor cells generated ex vivo." N Engl J Med 333(5): 283-7.

Carrier, R. L., M. Papadaki, M. Rupnick, F. J. Schoen, N. Bursac, R. Langer, L. E. Freed and G. Vunjak-Novakovic (1999). "Cardiac tissue engineering: cell seeding, cultivation parameters, and tissue construct characterization." Biotechnol Bioeng 64(5): 580-9.

Cascone, M. G., N. Barbani, C. Cristallini, P. Giusti, G. Ciardelli and L. Lazzeri (2001). "Bioartificial polymeric materials based on polysaccharides." J Biomater Sci Polym Ed 12(3): 267-81.

Cassell, O. C., W. A. Morrison, A. Messina, A. J. Penington, E. W. Thompson, G. W. Stevens, J. M. Perera, H. K. Kleinman, J. V. Hurley, R.

Romeo and K. R. Knight (2001). "The influence of extracellular matrix on the generation of vascularized, engineered, transplantable tissue." Ann N Y Acad Sci 944: 429-42.

Chabannon, C., J. L. Blache, I. Sielleur, J. Douville, C. Faucher, G. Gravis, C. Arnoulet, S. Oziel-Taieb, D. Blaise, G. Novakovitch, J. Camerlo, I. Chabbert, D. Genre, M. Appel, D. Armstrong, D. Maraninchi and P. Viens (1999a). "Production of ex vivo expanded hematopoietic cells and progenitors in a closed bioreactor, starting with a small volume marrow collection: A feasibility study in patients with poor-risk breast cancer and receiving high-doses of cyclophosphamide." Int.J.Oncol. 15(3): 511-518.

Chabannon, C., G. Novakovitch, J. L. Blache, S. Olivero, J. Camerlo, D. Genre, D. Maraninchi and P. Viens (1999b). "The role of autologous hematopoietic progenitor and cell reinfusion for intensive chemotherapy in women with poor-prognosis breast cancer. Clinical studies with ex-vivo expanded cells produced with the Aastrom Replicell technology." Hematol.Cell Ther. 41(2): 78-81.

Chachques, J. C., A. Shafy, F. Duarte, B. Cattadori, N. Goussef, L. Shen and A. Carpentier (2002). "From dynamic to cellular cardiomyoplasty." J Card Surg 17(3): 194-200.

Chown, S. R., M. N. Potter, J. Cornish, P. Goulden, N. Goulden, D. Pamphilon, C. G. Steward and A. O. Oakhill (1996). "Matched and mismatched unrelated donor bone marrow transplantation for juvenile chronic myeloid leukaemia." Br.J.Haematol. 93(3): 674-676.

Christensen, R., U. B. Jensen and T. G. Jensen (2002). "Skin genetically engineered as a bioreactor or a 'metabolic sink'." Cells Tissues Organs 172(2): 96-104.

Cohen, I., D. Robinson, N. Cohen and Z. Nevo (2000). "Storing live embryonic and adult human cartilage grafts for transplantation using a joint simulating device." Biomaterials 21(21): 2117-23.

Collins, P. C., W. M. Miller and E. T. Papoutsakis (1998a). "Stirred culture of peripheral and cord blood hematopoietic cells offers advantages over traditional static systems for clinically relevant applications." Biotechnol Bioeng 59(5): 534-43.

Collins, P. C., L. K. Nielsen, S. D. Patel, E. T. Papoutsakis and W. M. Miller (1998b). "Characterization of hematopoietic cell expansion, oxygen uptake,

and glycolysis in a controlled, stirred-tank bioreactor system." Biotechnol Prog 14(3): 466-72.

Cotter, T. G., R. S. Fernandes, S. Verhaegen and J. V. McCarthy (1994). "Cell death in the myeloid lineage." Immunol Rev **142**: 93-112.

Currie, L. J., J. R. Sharpe and R. Martin (2001). "The use of fibrin glue in skin grafts and tissue-engineered skin replacements: a review." Plast Reconstr Surg **108**(6): 1713-26.

Danet, G. H., Y. Pan, J. L. Luongo, D. A. Bonnet and M. C. Simon (2003). "Expansion of human SCID-repopulating cells under hypoxic conditions." J Clin Invest **112**(1): 126-35.

Dar, A., M. Shachar, J. Leor and S. Cohen (2002). "Optimization of cardiac cell seeding and distribution in 3D porous alginate scaffolds." Biotechnol Bioeng **80**(3): 305-12.

Dybedal, I. and S. E. Jacobsen (1995). "Transforming growth factor beta (TGF-beta), a potent inhibitor of erythropoiesis: neutralizing TGF-beta antibodies show erythropoietin as a potent stimulator of murine burst-forming unit erythroid colony formation in the absence of a burst-promoting activity." Blood **86**(3): 949-57.

Geer, D. J., D. D. Swartz and S. T. Andreadis (2002). "Fibrin promotes migration in a three-dimensional in vitro model of wound regeneration." Tissue Eng **8**(5): 787-98.

Gehling, U. M., J. W. Ryder, C. J. Hogan, L. Hami, I. McNiece, W. Franklin, S. Williams, K. Helm, J. King and E. J. Shpall (1997). "Ex vivo expansion of megakaryocyte progenitors: effect of various growth factor combinations on CD34+ progenitor cells from bone marrow and G-CSF-mobilized peripheral blood." Exp.Hematol. **25**(11): 1125-1139.

Grassl, E. D., T. R. Oegema and R. T. Tranquillo (2002). "Fibrin as an alternative biopolymer to type-I collagen for the fabrication of a media equivalent." J Biomed Mater Res **60**(4): 607-12.

Griffith, L. G. and G. Naughton (2002). "Tissue engineering--current challenges and expanding opportunities." Science **295**(5557): 1009-14.

Groger, A., S. Klaring, H. A. Merten, J. Holste, C. Kaps and M. Sittinger (2003). "Tissue engineering of bone for mandibular augmentation in

immunocompetent minipigs: preliminary study." Scand J Plast Reconstr Surg Hand Surg 37(3): 129-33.

Guerriero, A., L. Worford, H. K. Holland, G. R. Guo, K. Sheehan and E. K. Waller (1997). "Thrombopoietin is synthesized by bone marrow stromal cells." Blood **90**(9): 3444-55.

Haisch, A., S. Klaring, A. Groger, C. Gebert and M. Sittinger (2002). "A tissue-engineering model for the manufacture of auricular-shaped cartilage implants." Eur Arch Otorhinolaryngol **259**(6): 316-21.

Hildebrand, G., S. Kunze and M. Driver (2001). "Blood cell adhesion on sensor materials studied by light, scanning electron, and atomic-force microscopy." Ann Biomed Eng **29**(12): 1100-5.

Hoffman, A. S. (2001). "Hydrogels for biomedical applications." Ann N Y Acad Sci **944**: 62-73.

Hoffman, R., J. Tong, J. Brandt, C. Traycoff, E. Bruno, B. W. McGuire, M. S. Gordon, I. McNiece and E. F. Srour (1993). "The in vitro and in vivo effects of stem cell factor on human hematopoiesis." Stem Cells **11 Suppl 2**: 76-82.

Hojo, M., S. Inokuchi, M. Kidokoro, N. Fukuyama, E. Tanaka, C. Tsuji, M. Miyasaka, R. Tanino and H. Nakazawa (2003). "Induction of vascular endothelial growth factor by fibrin as a dermal substrate for cultured skin substitute." Plast Reconstr Surg **111**(5): 1638-45.

Huang, Q., J. C. Goh, D. W. Hutmacher and E. H. Lee (2002). "In vivo mesenchymal cell recruitment by a scaffold loaded with transforming growth factor beta1 and the potential for in situ chondrogenesis." Tissue Eng **8**(3): 469-82.

Jaroscak, J., K. Goltry, A. Smith, B. Waters-Pick, P. L. Martin, T. Driscoll, R. Howrey, N. Chao, J. Douville, S. Burhop, P. Fu and J. Kurtzberg (2003a). "Augmentation of umbilical cord blood (UCB) transplantation with ex-vivo expanded UCB cells: results of a phase I trial using the Aastrom Replicell system." Blood.

Jaroscak, J., K. Goltry, A. Smith, B. Waters-Pick, P. L. Martin, T. A. Driscoll, R. Howrey, N. Chao, J. Douville, S. Burhop, P. Fu and J. Kurtzberg (2003b). "Augmentation of umbilical cord blood (UCB) transplantation with ex vivo-expanded UCB cells: results of a phase 1 trial using the AastromReplicell System." Blood **101**(12): 5061-7.

Kapelushnik, J., G. Varadi and A. Nagler (1998). "Matched unrelated human umbilical cord blood transplantation for X-linked adrenoleukodystrophy." J Pediatr Hematol Oncol **20**(3): 257-9.

Karp, J. M., K. Rzeszutek, M. S. Shoichet and J. E. Davies (2003). "Fabrication of precise cylindrical three-dimensional tissue engineering scaffolds for in vitro and in vivo bone engineering applications." J Craniofac Surg **14**(3): 317-23.

Kawamoto, A., T. Tkebuchava, J. Yamaguchi, H. Nishimura, Y. S. Yoon, C. Milliken, S. Uchida, O. Masuo, H. Iwaguro, H. Ma, A. Hanley, M. Silver, M. Kearney, D. W. Losordo, J. M. Isner and T. Asahara (2003). "Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia." Circulation **107**(3): 461-8.

Knutsen, G., E. Solheim and O. Johansen (1998). "[Treatment of focal cartilage injuries in the knee]." Tidsskr Nor Laegeforen **118**(16): 2493-7.

Kogler, G., J. Callejas, R. V. Sorg and P. Wernet (1998). "An eight-fold ex vivo expansion of long-term culture-initiating cells from umbilical cord blood in stirred suspension cultures." Bone Marrow Transplant **21 Suppl 3**: S48-53.

Koller, M. R., J. G. Bender, W. M. Miller and E. T. Papoutsakis (1993a). "Expansion of primitive human hematopoietic progenitors in a perfusion bioreactor system with IL-3, IL-6, and stem cell factor." Biotechnology (N Y) **11**(3): 358-63.

Koller, M. R., S. G. Emerson and B. O. Palsson (1993b). "Large-scale expansion of human stem and progenitor cells from bone marrow mononuclear cells in continuous perfusion cultures." Blood **82**(2): 378-84.

Koller, M. R., I. Manchel, R. J. Maher, K. L. Goltry, R. D. Armstrong and A. K. Smith (1998). "Clinical-scale human umbilical cord blood cell expansion in a novel automated perfusion culture system." Bone Marrow Transplant **21**(7): 653-63.

Koller, M. R., I. Manchel, B. S. Newsom, M. A. Palsson and B. O. Palsson (1995). "Bioreactor expansion of human bone marrow: comparison of unprocessed, density-separated, and CD34-enriched cells." J Hematother **4**(3): 159-69.

Kurtzberg, J., M. Laughlin, M. L. Graham, C. Smith, J. F. Olson, E. C. Halperin, G. Ciocci, C. Carrier, C. E. Stevens and P. Rubinstein (1996). "Placental blood as a source of hematopoietic stem cells for transplantation into unrelated recipients." N.Engl.J.Med. **335**(3): 157-166.

LaIuppa, J. A., T. A. McAdams, E. T. Papoutsakis and W. M. Miller (1997). "Culture materials affect ex vivo expansion of hematopoietic progenitor cells." J Biomed Mater Res **36**(3): 347-59.

Laughlin, M. J., J. Barker, B. Bambach, O. N. Koc, D. A. Rizzieri, J. E. Wagner, S. L. Gerson, H. M. Lazarus, M. Cairo, C. E. Stevens, P. Rubinstein and J. Kurtzberg (2001). "Hematopoietic engraftment and survival in adult recipients of umbilical-cord blood from unrelated donors." N.Engl.J.Med. **344**(24): 1815-1822.

Leor, J., S. Aboulafia-Etzion, A. Dar, L. Shapiro, I. M. Barbash, A. Battler, Y. Granot and S. Cohen (2000). "Bioengineered cardiac grafts: A new approach to repair the infarcted myocardium?" Circulation **102**(19 Suppl 3): III56-61.

Linenberger, M. L., F. W. Jacobson, L. G. Bennett, V. C. Broudy, F. H. Martin and J. L. Abkowitz (1995). "Stem cell factor production by human marrow stromal fibroblasts." Exp Hematol **23**(10): 1104-14.

Lisovsky, M., S. E. Braun, Y. Ge, H. Takahira, L. Lu, V. G. Savchenko, S. D. Lyman and H. E. Broxmeyer (1996). "Flt3-ligand production by human bone marrow stromal cells." Leukemia **10**(6): 1012-8.

Low, H. P., T. M. Savarese and W. J. Schwartz (2001). "Neural precursor cells form rudimentary tissue-like structures in a rotating-wall vessel bioreactor." In Vitro Cell Dev Biol Anim **37**(3): 141-7.

Mackin, W., K. C. Gunter and M. Kraus (2001). "The frontier of cellular medicine: expanding umbilical cord blood stem cells for clinical use." Cancer J **7 Suppl 2**: S95-105.

Mandalam, R. K. and A. K. Smith (2002). "Ex vivo expansion of bone marrow and cord blood cells to produce stem and progenitor cells for hematopoietic reconstitution." Mil Med **167**(2 Suppl): 78-81.

Manosroi, J., C. Tayapiwatana, A. Manosroi, J. Beer, K. Bergemann and R. G. Werner (2002). "Lekteplase--a secreted tissue plasminogen activator derivative from Escherichia coli." Arzneimittelforschung **52**(1): 60-6.

Mantalaris, A., P. Keng, P. Bourne, A. Y. Chang and J. H. Wu (1998). "Engineering a human bone marrow model: a case study on ex vivo erythropoiesis." Biotechnol Prog **14**(1): 126-33.

McAdams, T. A., W. M. Miller and E. T. Papoutsakis (1996a). "Hematopoietic cell culture therapies (Part I): Cell culture considerations." Trends Biotechnol 14(9): 341-9.

McAdams, T. A., J. N. Winter, W. M. Miller and E. T. Papoutsakis (1996b). "Hematopoietic cell culture therapies (Part II): Clinical aspects and applications." Trends Biotechnol **14**(10): 388-96.

McDowell, C. L. and E. T. Papoutsakis (1998). "Increased agitation intensity increases CD13 receptor surface content and mRNA levels, and alters the metabolism of HL60 cells cultured in stirred tank bioreactors." Biotechnol Bioeng **60**(2): 239-50.

McNiece, I. and R. Briddell (2001). "Ex vivo expansion of hematopoietic progenitor cells and mature cells." Exp.Hematol. **29**(1): 3-11.

McNiece, I., R. Jones, S. I. Bearman, P. Cagnoni, Y. Nieto, W. Franklin, J. Ryder, A. Steele, J. Stoltz, P. Russell, J. McDermitt, C. Hogan, J. Murphy and E. J. Shpall (2000a). "Ex vivo expanded peripheral blood progenitor cells provide rapid neutrophil recovery after high-dose chemotherapy in patients with breast cancer." Blood **96**(9): 3001-3007.

McNiece, I., R. Jones, P. Cagnoni, S. Bearman, Y. Nieto and E. J. Shpall (1999). "Ex-vivo expansion of hematopoietic progenitor cells: preliminary results in breast cancer." Hematol.Cell Ther. **41**(2): 82-86.

McNiece, I., D. Kubegov, P. Kerzic, E. J. Shpall and S. Gross (2000b). "Increased expansion and differentiation of cord blood products using a two-step expansion culture." Exp.Hematol. **28**(10): 1181-1186.

McNiece, I. K. (2001). "Ex vivo expansion of hematopoietic cells: what is the clinical need?" J.Hematother.Stem Cell Res. **10**(3): 431-433.

Menasche, P. (2002). "Cell therapy of heart failure." C R Biol **325**(6): 731-8.

Murohara, T. (2003). "[Therapeutic angiogenesis by autologous cell implantation]." Nippon Rinsho **61**(3): 485-93.

Murray, L. J., J. C. Young, L. J. Osborne, K. M. Luens, R. Scollay and B. L. Hill (1999). "Thrombopoietin, flt3, and kit ligands together suppress apoptosis of human mobilized CD34+ cells and recruit primitive CD34+ Thy-1+ cells into rapid division." Exp Hematol **27**(6): 1019-28.

Negrin, R. S., K. Atkinson, T. Leemhuis, E. Hanania, C. Juttner, K. Tierney, W. W. Hu, L. J. Johnston, J. A. Shizurn, K. E. Stockerl-Goldstein, K. G. Blume, I. L. Weissman, S. Bower, R. Baynes, R. Dansey, C. Karanes, W. Peters and J. Klein (2000). "Transplantation of highly purified CD34+Thy-1+ hematopoietic stem cells in patients with metastatic breast cancer." Biol.Blood Marrow Transplant. **6**(3): 262-271.

Neovius, E. B. and G. Kratz (2003). "Tissue engineering by cocultivating human elastic chondrocytes and keratinocytes." Tissue Eng **9**(2): 365-9.

Nielsen, L. K. (1999). "Bioreactors for hematopoietic cell culture." Annu Rev Biomed Eng **1**: 129-52.

Noll, T., N. Jelinek, S. Schmid, M. Biselli and C. Wandrey (2002). "Cultivation of hematopoietic stem and progenitor cells: biochemical engineering aspects." Adv Biochem Eng Biotechnol **74**: 111-28.

Papadaki, M. (2001). "Promises and challenges in tissue engineering." IEEE Eng Med Biol Mag **20**(1): 117, 126.

Patel, S. D., E. T. Papoutsakis, J. N. Winter and W. M. Miller (2000). "The lactate issue revisited: novel feeding protocols to examine inhibition of cell proliferation and glucose metabolism in hematopoietic cell cultures." Biotechnol Prog **16**(5): 885-92.

Peled, T., E. Landau, E. Prus, A. J. Treves and E. Fibach (2002). "Cellular copper content modulates differentiation and self-renewal in cultures of cord blood-derived CD34+ cells." Br.J.Haematol. **116**(3): 655-661.

Pereira, R. F., M. D. O'Hara, A. V. Laptev, K. W. Halford, M. D. Pollard, R. Class, D. Simon, K. Livezey and D. J. Prockop (1998). "Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta." Proc Natl Acad Sci U S A **95**(3): 1142-7.

Piacibello, W., F. Sanavio, L. Garetto, A. Severino, D. Bergandi, J. Ferrario, F. Fagioli, M. Berger and M. Aglietta (1997). "Extensive amplification and self-renewal of human primitive hematopoietic stem cells from cord blood." Blood **89**(8): 2644-53.

Pittenger, M. F., A. M. Mackay, S. C. Beck, R. K. Jaiswal, R. Douglas, J. D. Mosca, M. A. Moorman, D. W. Simonetti, S. Craig and D. R. Marshak (1999).

"Multilineage potential of adult human mesenchymal stem cells." Science 284(5411): 143-7.

Purdy, M. H., C. J. Hogan, L. Hami, I. McNiece, W. Franklin, R. B. Jones, S. I. Bearman, R. J. Berenson, P. J. Cagnoni and S. Heimfeld (1995). "Large volume ex vivo expansion of CD34-positive hematopoietic progenitor cells for transplantation." J.Hematother. 4(6): 515-525.

Rafii, S. and D. Lyden (2003). "Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration." Nat Med 9(6): 702-12.

Ramsfjell, V., D. Bryder, H. Bjorgvinsdottir, S. Kornfalt, L. Nilsson, O. J. Borge and S. E. Jacobsen (1999). "Distinct requirements for optimal growth and In vitro expansion of human CD34(+)CD38(-) bone marrow long-term culture-initiating cells (LTC-IC), extended LTC-IC, and murine in vivo long-term reconstituting stem cells." Blood 94(12): 4093-102.

Richel, D. J., H. E. Johnsen, J. Canon, T. Guillaume, M. R. Schaafsma, C. Schenkeveld, S. W. Hansen, I. McNiece, A. J. Gringeri, R. Briddell, C. Ewen, R. Davies, J. Freeman, S. Miltenyi and M. Symann (2000). "Highly purified CD34+ cells isolated using magnetically activated cell selection provide rapid engraftment following high-dose chemotherapy in breast cancer patients." Bone Marrow Transplant. 25(3): 243-249.

Risbud, M. V. and M. Sittinger (2002). "Tissue engineering: advances in in vitro cartilage generation." Trends Biotechnol 20(8): 351-6.

Robinson, D., H. Ash, A. Yayon, Z. Nevo and D. Aviezer (2001). "Characteristics of cartilage biopsies used for autologous chondrocytes transplantation." Cell Transplant 10(2): 203-8.

Robinson, D., A. Hasharoni, N. Cohen, A. Yayon, R. M. Moskowitz and Z. Nevo (1999). "Fibroblast growth factor receptor-3 as a marker for precartilaginous stem cells." Clin Orthop(367 Suppl): S163-75.

Sakai, T., R. K. Li, R. D. Weisel, D. A. Mickle, Z. Q. Jia, S. Tomita, E. J. Kim and T. M. Yau (1999). "Fetal cell transplantation: a comparison of three cell types." J Thorac Cardiovasc Surg 118(4): 715-24.

Sandstrom, C. E., J. G. Bender, E. T. Papoutsakis and W. M. Miller (1995). "Effects of CD34+ cell selection and perfusion on ex vivo expansion of peripheral blood mononuclear cells." Blood 86(3): 958-70.

Sandstrom, C. E., P. C. Collins, T. A. McAdams, J. G. Bender, E. T. Papoutsakis and W. M. Miller (1996). "Comparison of whole serum-deprived media for ex vivo expansion of hematopoietic progenitor cells from cord blood and mobilized peripheral blood mononuclear cells." J Hematother 5(5): 461-73.

Sardonini, C. A. and Y. J. Wu (1993). "Expansion and differentiation of human hematopoietic cells from static cultures through small-scale bioreactors." Biotechnol Prog 9(2): 131-7.

Schwartz, R. M., B. O. Palsson and S. G. Emerson (1991). "Rapid medium perfusion rate significantly increases the productivity and longevity of human bone marrow cultures." Proc Natl Acad Sci U S A 88(15): 6760-4.

Sen, A., M. S. Kallos and L. A. Behie (2002a). "Expansion of mammalian neural stem cells in bioreactors: effect of power input and medium viscosity." Brain Res Dev Brain Res 134(1-2): 103-13.

Sen, A., M. S. Kallos and L. A. Behie (2002b). "Passaging protocols for mammalian neural stem cells in suspension bioreactors." Biotechnol Prog 18(2): 337-45.

Shapiro, L. and S. Cohen (1997). "Novel alginate sponges for cell culture and transplantation." Biomaterials 18(8): 583-90.

Shizuru, J. A., L. Jerabek, C. T. Edwards and I. L. Weissman (1996). "Transplantation of purified hematopoietic stem cells: requirements for overcoming the barriers of allogeneic engraftment." Biol Blood Marrow Transplant 2(1): 3-14.

Shpall, E. J., R. Quinones, R. Giller, R. B. Jones, S. I. Bearman, P. J. Cagnoni, Y. Nieto, P. McSweeney, J. Murphy, C. Hogan, A. Gocke, W. Ward, V. Slat-Vasquez, P. Russel, B. Blunk, M. Solzan and I. K. McNiece (2000). "Transplantation of Adult and Pediatric Cancer Patients with Cord Blood Progenitors Expanded ex vivo." Blood 96(11 (1)): 207a.

Shur, I., F. Lokiec, I. Bleiberg and D. Benayahu (2001). "Differential gene expression of cultured human osteoblasts." J Cell Biochem 83(4): 547-53.

Shur, I., R. Marom, F. Lokiec, R. Socher and D. Benayahu (2002). "Identification of cultured progenitor cells from human marrow stroma." J Cell Biochem 87(1): 51-7.

Sodian, R., S. P. Hoerstrup, J. S. Sperling, S. Daebritz, D. P. Martin, A. M. Moran, B. S. Kim, F. J. Schoen, J. P. Vacanti and J. E. Mayer, Jr. (2000).

"Early in vivo experience with tissue-engineered trileaflet heart valves." Circulation 102(19 Suppl 3): III22-9.

Sodian, R., M. Loebe, A. Hein, D. P. Martin, S. P. Hoerstrup, E. V. Potapov, H. Hausmann, T. Lueth and R. Hetzer (2002). "Application of stereolithography for scaffold fabrication for tissue engineered heart valves." Asaio J 48(1): 12-6.

Spitzer, R. S., C. Perka, K. Lindenhayn and H. Zippel (2002). "Matrix engineering for osteogenic differentiation of rabbit periosteal cells using alpha-tricalcium phosphate particles in a three-dimensional fibrin culture." J Biomed Mater Res 59(4): 690-6.

Tock, B., W. Drohan, J. Hess, A. Pusateri, J. Holcomb and M. MacPhee (1998). "Haemophilia and advanced fibrin sealant technologies." Haemophilia 4(4): 449-55.

Vilquin, J. T., J. P. Marolleau, A. Hagege, P. Menasche, M. Fiszman and K. Schwartz (2002). "Cell transplantation for post-ischemic heart failure." Arch Mal Coeur Vaiss 95(12): 1219-25.

Wagner, J. E., Jr. (1993). "Umbilical cord blood stem cell transplantation: current status and future prospects (1992)." J.Hematother. 2(2): 225-228.

Wagner, J. E., J. Rosenthal, R. Sweetman, X. O. Shu, S. M. Davies, N. K. Ramsay, P. B. McGlave, L. Sender and M. S. Cairo (1996). "Successful transplantation of HLA-matched and HLA-mismatched umbilical cord blood from unrelated donors: analysis of engraftment and acute graft-versus-host disease." Blood 88(3): 795-802.

Wakitani, S., T. Saito and A. I. Caplan (1995). "Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine." Muscle Nerve 18(12): 1417-26.

Wechselberger, G., R. C. Russell, M. W. Neumeister, T. Schoeller, H. Piza-Katzer and C. Rainer (2002). "Successful transplantation of three tissue-engineered cell types using capsule induction technique and fibrin glue as a delivery vehicle." Plast Reconstr Surg 110(1): 123-9.

Williams, S. F., D. P. Martin, D. M. Horowitz and O. P. Peoples (1999). "PHA applications: addressing the price performance issue: I. Tissue engineering." Int J Biol Macromol 25(1-3): 111-21.

Wolff, S. N. (2002). "Second hematopoietic stem cell transplantation for the treatment of graft failure, graft rejection or relapse after allogeneic transplantation." Bone Marrow Transplant 29(7): 545-52.

Zandstra, P. W., C. J. Eaves and J. M. Piret (1994). "Expansion of hematopoietic progenitor cell populations in stirred suspension bioreactors of normal human bone marrow cells." Biotechnology (N Y) 12(9): 909-14.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

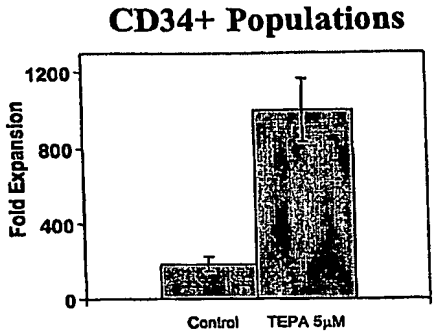
Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

Claims:

1. Methods for ex-vivo or in-vivo expanding stem and/or progenitor cells essentially as described and exemplified herein.

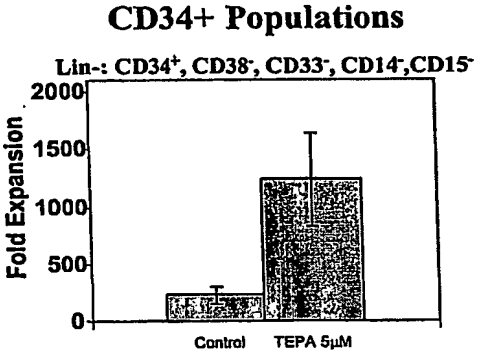
Figure 1

A



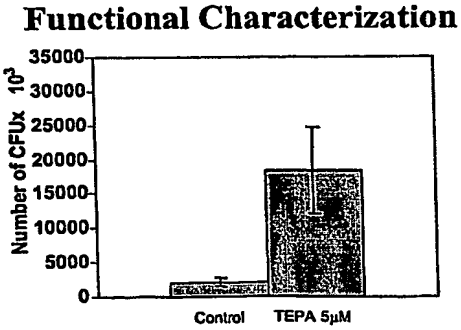
CD34+/38- Sub-Population: Treatment Phase (3 weeks)

B



CD34+/Lin- Sub-Population: Treatment Phase (3 weeks)

C



Long Term Potential of CD34+ Cell Cultures: LTC-CFC (7-12 weeks)

Figure 1 Copper chelation by 5 μM TEPA tremendously increases the proportional portion of immature HSC ($CD34^+/CD38^-$ and $CD34^+/lin^-$ cells) as compared to control conditions. These immature subpopulations are indicative of the long term potential of the culture and thus provide a good representation of the ex vivo expansion of the culture. See the text for further details.